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**Patentanmeldung Nr. | Patent application No. | Demande de brevet n°**

02028880.9

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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

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Axxima Pharmaceuticals Aktiengesellschaft
Am Klopferspitz 19
82152 Martinsried
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(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Medium and method for enriching, purifying or depleting ATP binding proteins from a pool of proteins

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# Medium and method for enriching, purifying or depleting ATP binding proteins from a pool of proteins

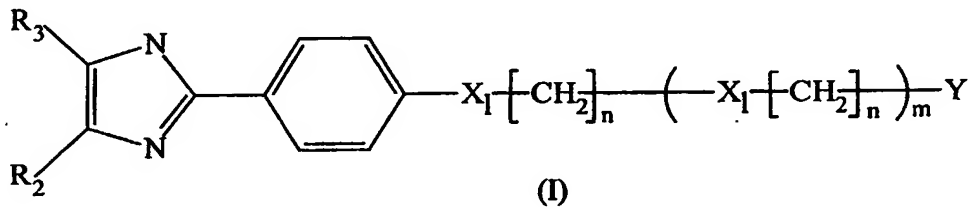
The present invention refers to a medium and a method for enriching, purifying or depleting ATP binding proteins from a pool of proteins, such as a proteome.

ATP binding proteins play an important role in the metabolism of an organism. E.g., enzymes of the protein kinase family are essential switches of the cellular signal transduction machinery in all eucaryotic cells. They have been implicated with the control of numerous physiological and pathophysiological processes in eucaryotic organisms and therefore represent an important class of drug targets for a variety of indications such as cancer, inflammation and infectious diseases. Biochemical identification of protein kinases relevant for disease progression has been a rather difficult methodological challenge in the past and there is a clear need for novel and innovative techniques which allow rapid and systematic biochemical analysis of all cellular kinase activities. The most efficient established techniques for parallel analysis of cellular proteins are two-dimensional gel electrophoresis in combination with mass spectrometry for identification of separated protein spots. But due to the enormous complexity of the proteome of an individual, this approach has not been successful for identification of protein kinase targets, since most protein kinases are low abundance proteins that are not detectable if unfractionated cellular extracts are used for proteome analysis. Thus, efficient and selective enrichment is a prerequisite for subsequent identification of protein kinase targets by a proteomics approach. As no efficient pre-fractionation techniques have been reported to date, novel experimental approaches are required to accomplish these tasks.

It is therefore the object of the present invention to provide a medium and a method which are capable of enriching, purifying or depleting ATP binding proteins from a pool of proteins, such as a proteome, a cell lysate or a tissue lysate.

This object is solved by the medium according to independent claim 1 and the method according to independent claim 12. Further advantageous features, aspects, and details of the invention are evident from the dependent claims, the description, the examples, and the drawings.

According to one aspect, the present invention relates to a medium for separating at least one ATP binding protein from a pool of proteins, like a proteome of an individual, the medium comprising at least one compound of the following formula I (compound class A)



(I)

wherein

X is $-CH_2-$, -NH-, -O-, or –S-,

Y is $-NH_2$, $-NHR_1$, -OH or –SH,

l is 0 or 1,

m is an integer from 0 to 10,

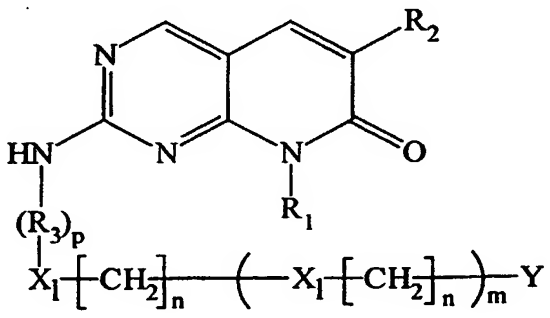
n is an integer from 0 to 10,

$R_1$ is $C_1 - C_6$ alkyl (linear or branched) or aryl,

$R_2$ is unsubstituted or partially or fully substituted aryl, substituted by -F, -Cl, -Br, -I, -CN, -OH, -SH- , $C_1 - C_6$-alkoxy, $C_1 - C_6$ -alkylthio, $C_1 - C_6$ -haloalkyloxy, $C_1$-$C_6$ haloalkyl ($C_1 - C_6$-alkoxy denotes an O-alkyl group, $C_1 - C_6$-alkylthio denotes an S-alkyl group, $C_1 - C_6$-haloalkyloxy denotes an halogen-alkyl-O group, $C_1 - C_6$-haloalkyl denotes an halogen-alkyl group wherein the alkyl group is linear or branched), and

$R_3$ is pyridinyl or pyrimidinyl;

the following formula II (compound class B)



(II)

wherein

$R_1$ is $C_1 - C_6$ alkyl (linear or branched) or aryl,

$R_2$ and $R_3$ are unsubstituted or partially or fully substituted aryl substituted by -F, -Cl, -Br, -I, -CN, -OH, -SH-, $C_1 - C_6$-alkoxy, $C_1 - C_6$ -alkylthio, $C_1 - C_6$ -haloalkyloxy, $C_1$-$C_6$ haloalkyl ($C_1 - C_6$-alkoxy denotes an O-alkyl group, $C_1 - C_6$-alkylthio denotes an S-alkyl group, $C_1 - C_6$-haloalkyloxy denotes an halogen-alkyl-O group, $C_1 - C_6$-haloalkyl denotes an halogen-alkyl group wherein the alkyl group is linear or branched),X is $-CH_2-$,-NH-, -O-, -S-,

Y is $-NH_2$, $-NHR_1$, -OH or –SH,

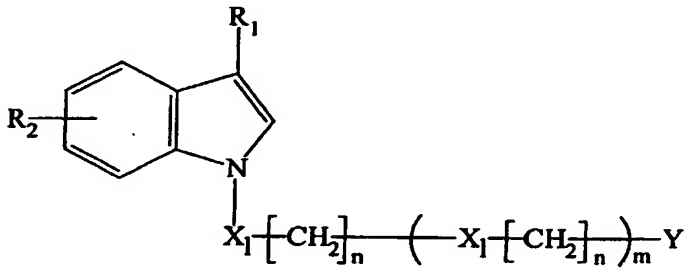
l is 0 or 1,

m is an integer from 0 to 10,

n is an integer from 0 to 10, and

p is an integer from 0 to 2;

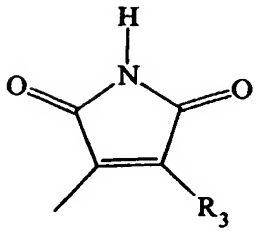
the following formula III (compound class C)



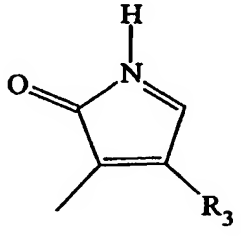
(III)

wherein

$R_1$ is



or



$R_3$ is -indolyl, N-alkyl-indolyl, $-NHR_1'$, $-S-R_1'$, $-O-R_1'$,

$R_1'$ is $C_1 - C_6$ alkyl (linear or branched) or aryl,

$R_2$ is -H, -F, -Cl, -Br, -I, -CN, -OH, -SH- , $C_1$ – $C_6$-alkoxy, $C_1$ – $C_6$ -alkylthio, $C_1$ – $C_6$ -haloalkyloxy, $C_1$ - $C_6$ haloalkyl ($C_1$ – $C_6$-alkoxy denotes an O-alkyl group, $C_1$ – $C_6$-alkylthio denotes an S-alkyl group, $C_1$ – $C_6$-haloalkyloxy denotes an halogen-alkyl-O group, $C_1$ – $C_6$-haloalkyl denotes an halogen-alkyl group wherein the alkyl group is linear or branched),

X is -$CH_2$-,-NH-, -O-, or –S-,

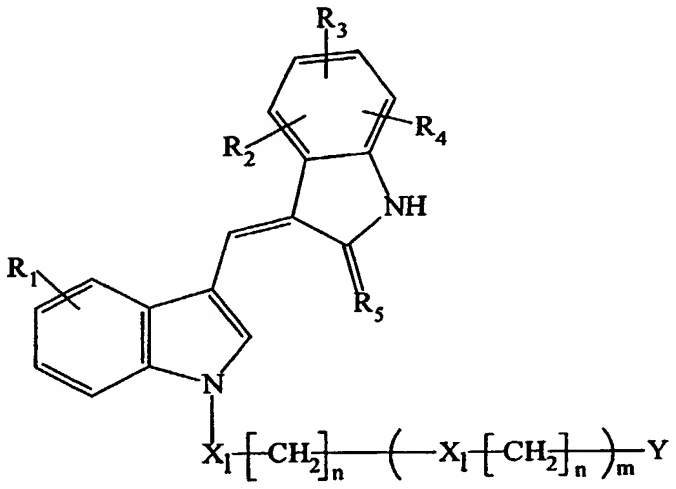
Y is -$NH_2$, -$NHR_1$, -OH or –SH,

l is 0 or 1,

m is an integer from 0 to 10, and

n is an integer from 0 to 10;

and the following formula IV (compound class D)



(IV)

wherein

X is -$CH_2$-,-NH-, -O-, or –S-,

Y is -$NH_2$, -NHR', -OH or –SH,

Z is -$CH_2$, -CO, -O-CO, -N-CO, -$OCH_2$, or -$SCH_2$, -CO-O, -CO-N

l is 0 or 1,

m is 0 – 10,

n is 0 – 10,

$R_1$ is -Z-NH-$(CH_2)_n$-$[X_l$-$(CH_2)_n]_m$-Y,

R' is $C_1$ – $C_6$ alkyl (linear or branched) or aryl,

$R_2$, $R_3$, and $R_4$ represent independently of each other -H, -F, -Cl, -Br, -I, -CN, -OH, -SH- , $C_1$ – $C_6$-alkoxy, $C_1$ – $C_6$ -alkylthio, $C_1$ – $C_6$ -haloalkyloxy, $C_1$-$C_6$ haloalkyl ($C_1$ – $C_6$-alkoxy

denotes an O-alkyl group, $C_1 - C_6$-alkylthio denotes an S-alkyl group, $C_1 - C_6$-haloalkyloxy denotes an halogen-alkyl-O group, $C_1 - C_6$-haloalkyl denotes an halogen-alkyl group wherein the alkyl group is linear or branched), and

$R_5$ is S or O.

It is preferred that the compounds of the compound classes A to D according the general formulas I to IV are covalently bound to the support material.

Particularly preferred are from the compound class A 4-[4-(4-fluoro-phenyl)-5-pyridine-4-yl-1*H*-imidazole-2-yl]-benzylamine ("compound A"), from the compound class B 2-[4-(amino-ethoxy)-phenylamino]-6-(2,6-dichloro-phenyl)-8-methyl-8*H*-pyrido[2,3-d]pyrimidine-7-one ("compound B"), from the compound class C 2-[1-(3-aminopropyl)-1*H*-indole-3-yl)maleimide ("compound C") and/or from the compound class D 3-(5-chloro-2-oxo-1,2-dihydro-indole-3-ylidenemethyl)-1*H*-indole-5-carboxylic acid (2-amino-ethyl)-amide ("compound D") immobilized on a support material.

According to a further preferred aspect, the support material comprises or consists of an agarose material, particularly a modified agarose-material like an epoxy-activated Sepharose 6B material (Sepharose is obtainable from Amersham Biosciences). It is especially preferred if the support material for the compound classes A to D is the modified agarose material referred to above.

According to a still further aspect, the support material comprises or consists of ferro- or ferrimagnetic particles as e.g. known from WO 01/71732, incorporated herein by reference as far as properties of ferro- or ferrimagnetic particles are concerned. The ferro- or ferrimagnetic particles may be made from glass or from plastic. The ferro- or ferrimagnetic particles that can be used with the present invention may be porous. The ferro- or ferrimagnetic glass particles may comprise about 30 to 50 % by weight of $Fe_3O_4$ and about 50 to 70 % by weight of $SiO_2$. The ferro- or ferrimagnetic particles used herein preferably have an average size of about 5 to 25 µm in diameter, more preferably about 6 to 15 µm, and particularly about 7 to 10 µm. The total surface area of the ferro- or ferrimagnetic particles may be 190 g/m² or greater, e.g. in the range of about 190 to 270 g/m² (as determined according the Brunaur Emmet Teller (BET) method).

These magnetic particles facilitate purification, separation and/or assay of biomolecules, like protein kinases. Magnetic particles (or beads) that bind a molecule of interest can be collected or retrieved by applying an external magnetic field to a container comprising the particles. Unbound molecules and supernatant liquid can be separated from the particles or discarded, and the molecules bound to the magnetic particles may be eluted in an enriched state.

Although in the following it is described that compounds of classes A to D (formulas I to IV) were used separately bound to the support material, it is clear that also any combination of the immobilized compounds can be used according to the present invention to enrich, purify or deplete ATP binding proteins from a pool of different proteins, like from a proteome.

According to another aspect, the present invention refers to a method for enriching, purifying or depleting at least one ATP binding protein, e.g. a protein kinase, from a pool of proteins containing at least one such ATP binding protein , the method comprising the following steps (a) immobilizing at least one of the compounds of the compound classes A to D (compounds of the formulas I to IV) as described above on a support material, (b) bringing the pool of proteins containing at least one ATP binding protein into contact with at least one of the immobilized compounds of the compound classes A to D (compounds of the formulas I to IV), and (c) separating the proteins not bound to the at least one compound of the compound classes A to D (compounds of the formulas I to IV) immobilized on the support material from the at least one ATP binding protein bound to the compound of the compound classes A to D (compounds of the formulas I to IV) immobilized on the support material.

According to still further preferred aspect, in step (a) at least one of the compounds 4-[4-(4-fluoro-phenyl)-5-pyridine-4-yl-1*H*-imidazole-2-yl]-benzylamine, 2-[4-(amino-ethoxy)-phenylamino]-6-(2,6-dichloro-phenyl)-8-methyl-8*H*-pyrido[2,3-d]pyrimidine-7-one, 2-[1-(3-aminopropyl)-1*H*-indole-3-yl)maleimide and 3-(5-chloro-2-oxo-1,2-dihydro-indole-3-ylidenemethyl)-1*H*-indole-5-carboxylic acid (2-amino-ethyl)-amide is immobilized on the support material; in step (b) the pool of proteins containing at least one ATP binding protein is brought into contact with at least one of the compounds 4-[4-(4-fluoro-phenyl)-5-pyridine-4-yl-1*H*-imidazole-2-yl]-benzylamine, 2-[4-(amino-ethoxy)-phenylamino]-6-(2,6-dichloro-phenyl)-8-methyl-8*H*-pyrido[2,3-d]pyrimidine-7-one, 2-[1-(3- aminopropyl)-1*H*-indole-3-yl)maleimide and 3-(5-chloro-2-oxo-1,2-dihydro-indole-3-ylidenemethyl)-1*H*-indole-5-carboxylic acid (2-amino-ethyl)-amide immobilized on the support material; and in step (c)

the proteins not bound to the at least one compound 4-[4-(4-fluoro-phenyl)-5-pyridine-4-yl-1*H*-imidazole-2-yl]-benzylamine, 2-[4-(amino-ethoxy)-phenylamino]-6-(2,6-dichloro-phenyl)-8-methyl-8*H*-pyrido[2,3-d]pyrimidine-7-one, 2-[1-(3-aminopropyl)-1*H*-indole-3-yl)maleimide and 3-(5-chloro-2- oxo-1,2-dihydro-indole-3-ylidenemethyl)-1*H*-indole-5-carboxylic acid (2-amino-ethyl)-amide immobilized on the support material are separated from the at least one ATP binding protein bound to the compound 4-[4-(4-fluoro-phenyl)-5-pyridine-4-yl-1*H*-imidazole-2-yl]-benzylamine, 2-[4-(amino-ethoxy)-phenylamino]-6-(2,6-dichloro-phenyl)-8-methyl-8*H*-pyrido [2,3-d]pyrimidine-7-one, 2-[1-(3-aminopropyl)-1*H*-indole-3-yl)maleimide and/or 3-(5-chloro-2-oxo-1,2-dihydro-indole-3-ylidenemethyl)-1*H*-indole-5-carboxylic acid (2-amino-ethyl)-amide immobilized on the support material.

According to a still further preferred embodiment, the method of the present invention comprises a further step (d) releasing the at least one ATP binding protein bound to the at least one compound of the compound classes A to D (formulas I to IV) immobilized on the support material from the at least one of said compounds. This releasing is preferably effected with a buffer containing the respective immobilized compound plus ATP (in this context it is clear that the "immobilized compound" contained in the releasing buffer is not the one fixed to the support material, but of course another amount of the same material).

According to a still further aspect, the method according to the present invention comprises further a step (e) collecting the at least one ATP binding protein released from the immobilized compound(s) of the compound classes A to D.

There were identified four structurally unrelated compound classes with primary amine substituents assumed to refer to the ATP binding sites of ATP binding proteins, like protein kinases, which have ideal properties for immobilization on solid support materials via the primary amines. These compound classes are the classes A to D represented by the general formulas I, II, III and IV described in detail above. Among those compounds falling under the general formulas I to IV, compound A, i.e. 4-[4-(4-Fluoro-phenyl)-5-pyridin-4-yl-1*H*-imidazol-2-yl]-benzylamine; compound B, i.e. 2-[4-(Amino-ethoxy)-phenylamino]-6-(2,6-dichloro-phenyl)-8-methyl-8*H*-pyrido[2,3-d]pyrimidin-7-one; compound C, i.e. 2-[1-(3-Aminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide; and compound D, i.e. 3-(5-Chloro-oxo-1,2-dihydro-indole-3-ylidenemethyl)-1*H*-indole-5-carboxylic acid(2-amino-ethyl)-amide are particularly preferred. Each of the compounds falling under the general

formulas I to IV can be coupled to a support material, e.g. a modified agarose material (e.g. epoxy-activated Sepharose 6B modified by reaction of the compounds' primary amines with the epoxy group of the 1,4-bis(2,3-epoxypropoxy)-butane spacer of the epoxy-activated Sepharose 6B beads) or the ferro- or ferrimagnetic particles described in more detail above. The coupling of the compounds of the compound classes A to D to the support material according to a preferred embodiment of the invention is covalently. The novel reagents (compounds A to D plus solid support material) are referred to in the following as Kinator I (containing immobilized compound A), Kinator II (containing immobilized compound B), Kinator III (containing immobilized compound C), and Kinator IV (containing immobilized compound D). Epoxy-activated Sepharose 6B was chosen as a preferred support material since it provides a long hydrophilic 12 atom spacer, thereby minimizing the risk of a sterical clash of a protein kinase bound to the immobilized inhibitor with the resin polymer of the support material.

Based on a variety of newly defined criteria for a novel selection scheme to identify suitable compounds for novel kinase target identification applications, there were identified four compound classes suitable for covalent coupling to a solid support material. By reacting the compounds A, B, C, and D falling under the general formulas I to IV with epoxy-activated Sepharose 6B, four novel reagents named Kinator I, II, III, and IV were generated that had not been reported before. The novel reagents have the ability to selectively bind sets of endogenously expressed cellular ATP binding proteins, like protein kinases, thereby efficiently enriching, purifying or depleting ATP binding proteins from total cell extracts. Furthermore, a novel elution procedure for affinity chromatography was developed on matrices containing bound ATP binding protein inhibitors, like protein kinase inhibitors, which depends on concomitant addition of both free compound (inhibitor) and ATP for quantitative ATP binding protein elution from the Kinator chromatography media and related chromatography media. Thus, the present invention relates to the conception and generation of these novel separation matrices and their application for the purpose of affinity purification of ATP binding proteins like protein kinases.

Due to the enormous complexity of the proteome, approaches to identify ATP binding protein targets have not been successful previously, since most of the ATP binding proteins are low abundance proteins that are not detectable if unfractionated cellular extracts are used for proteome analysis. Thus, efficient and selective enrichment and/or purification is a

prerequisite for subsequent identification of ATP binding protein targets, like protein kinase targets, by a proteomics approach. With the present invention, it is possible for the first time to selectively enrich or purify ATP binding proteins like protein kinases from a heterogeneous pool of proteins. As will be shown below, the concentration of ATP binding proteins can be increased by e.g. a hundred times using the medium and method according to the present invention. It was shown according to the present invention that the inventive media efficiently bind subsets of endogenously expressed ATP binding proteins by *in vitro* interaction studies. Furthermore, a novel elution protocol could be established which allowed specific elution of a representative ATP binding protein, such as a specific protein kinase, from the inventive media under non-denaturing conditions.

The buffer used to separate the bound ATP binding proteins from the proteins not bound preferably contains from 5 to 500 mM Hepes/NaOH pH 6.5 to 8.5 and/or 5 to 500 mM Tris-HCl pH 6.8 to 9.0, 0 to 1000 mM NaCl, 0 to 5 % Triton X-100, 0 to 500 mM EDTA, and 0 to 200 mM EGTA. If the buffer is used to release the bound ATP binding protein(s), it contains furthermore preferably 1 to 100 mM ATP, 1-200 mM $MgCl_2$ and 0.1 to 10 mM of at least one of the compounds of the compound classes A to D, particularly e.g. 4-[4-(4-fluoro-phenyl)-5-pyridine-4-yl-1*H*-imidazole-2-yl]-benzylamine, 2-[4-(amino-ethoxy)-phenylamino]-6-(2,6-dichloro-phenyl)-8-methyl-8*H*-pyrido [2,3-d]pyrimidine-7-one, 2-[1-(3-aminopropyl)-1*H*-indole-3-yl)maleimide and/or 3-(5-chloro-2- oxo-1,2-dihydro-indole-3-ylidenemethyl)-1*H*-indole-5-carboxylic acid (2-amino-ethyl)-amide.

Specifically, the ATP binding proteins, e.g. protein kinases, could be enriched from the pool of proteins used as the starting material with the medium or the method according to the present invention at least 100-fold, e.g. 100- to 1000-fold.

According to a particularly preferred embodiment of the present invention, the pool of proteins from which the at least one ATP binding protein is separated contains a high salt concentration. "High salt concentration" means according to the present invention a concentration of 0.5 to 5 M, preferably 0.5 to 3 M, more preferably from 0.75 to 2 M and particularly about 1 M. Every salt may be used which does not occupy the ATP binding site of the ATP binding protein. Some salts of alkaline earth metals, like magnesium chloride ($MgCl_2$), have a tendency to bind at the ATP binding site of respective protein, so that such salts are not preferred according to the present invention .On the other hand, e.g. alkali metal

salts do not compete with the ATP binding site of ATP binding proteins. Consequently, particularly preferred salts are salts of alkali metals, especially sodium chloride (NaCl). The buffer used to separate the ATP binding protein(s) bound to the novel reagents (Kinator I, II, III and/or IV) from the proteins not bound also may contain high salt concentrations in the above-mentioned sense.

Using such specific conditions, i.e. high salt concentration, allows enriching of ATP binding proteins at least $10^3$-fold, preferably at least $10^4$- fold, and more preferably up to $10^6$-fold.

Besides enriching, with the medium and the method according the present invention it is also possible to purify an ATP binding protein to a high degree, and vice versa, if one intends to deplete a pool of proteins specifically from ATP binding proteins, then this can also be achieved with the medium and the method according to the present invention.

The present invention also refers to a kit comprising at least of the mediums (compound of the classes A to D immobilized on a carrier) described in more detail above. The kit according the present invention may furthermore comprise one or more of the buffers described above.

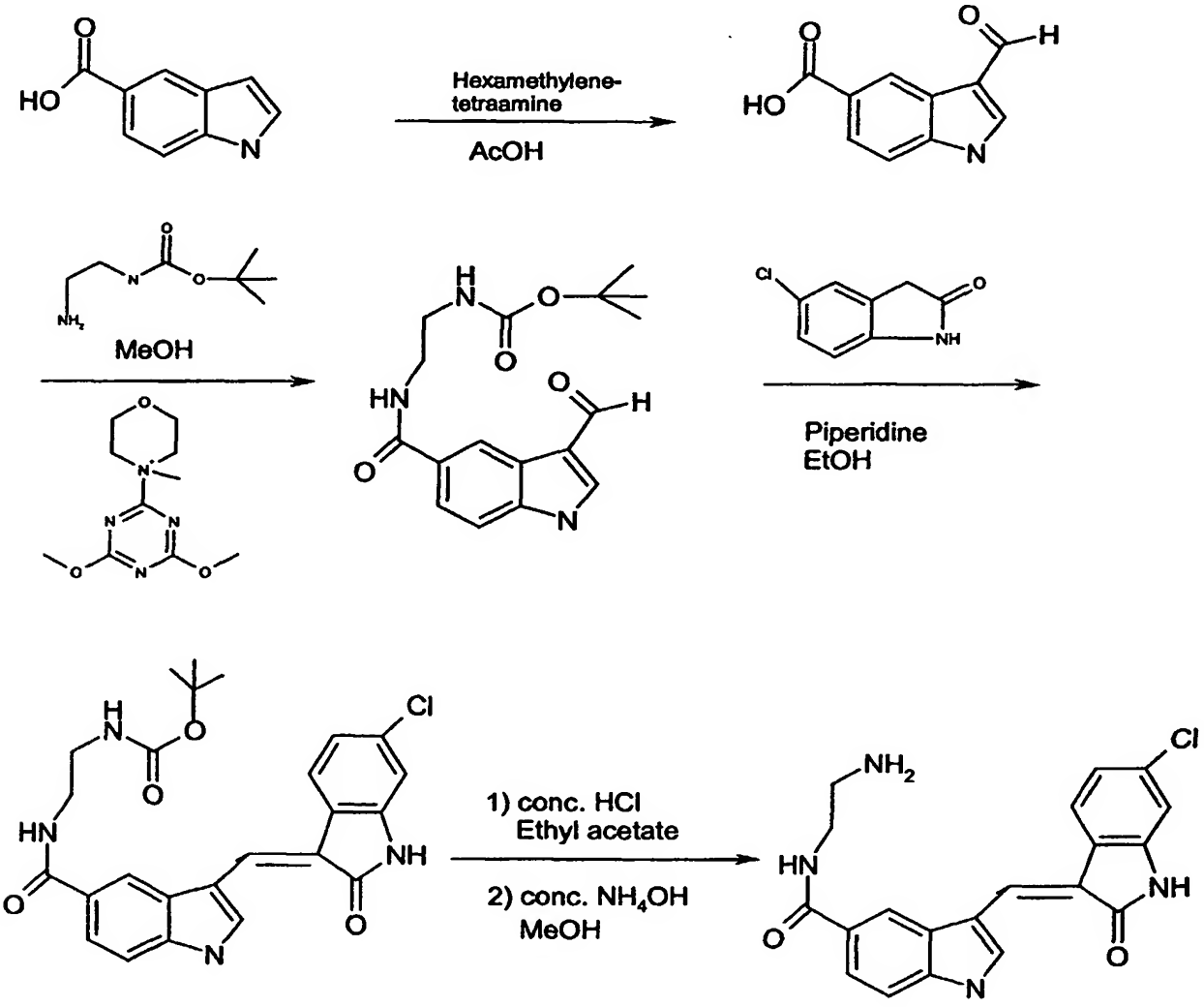
## Examples

Kinase inhibitors used and their immobilization

Protein kinase inhibitors used were compound A: 4-[4-(4-Fluoro-phenyl)-5-pyridin-4-yl-1*H*-imidazol-2-yl]-benzylamine (prepared as described in Gallagher et al., 1997, Bioorg. Med. Chem, 5, 49-64); compound B: 2-[4-(-Amino-ethoxy)-phenylamino]-6-(2,6-dichloro-phenyl)-8-methyl-8*H*-pyrido[2,3-d]pyrimidin-7-one (prepared as described in Klutschko et al., 1998, J. Med. Chem., 41, 3276-3292); compound C: 2-[1-(3-Aminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide (purchased from Calbiochem), and compound D: 3-(5-Chloro-oxo-1,2-dihydro-indole-3-ylidenemethyl)-1*H*-indole-5-carboxylic acid(2-amino-ethyl)-amide (synthesized as described below). 1 g epoxy-activated Sepharose 6B (Amersham Biosciences) was swollen and washed twice in 50 ml $H_2O$ and equilibrated to 50% DMF/0.1 M $Na_2CO_3$. Between all the washing steps, the Sepharose beads were spun down for 1 min at 2000 rpm in a desktop centrifuge and the supernatant was discarded. 300 µl drained beads were resuspended in 600 µl 20 mM compound A, B, C or D dissolved in 50% DMF/0.1 M $Na_2CO_3$. 1 µl 10 M NaOH was added followed by incubation overnight at 30°C with continual

agitation in the dark. After washing the beads three times in 1 ml 50% DMF/0.1 M $Na_2CO_3$ 600 µl 1 M ethanolamine were added to the drained beads and incubated for 6 h at 30°C with permanent shaking in the dark. Finally the following washing steps were carried out in a volume of 1 ml each: First 50% DMF/0.1 M $Na_2CO_3$, then $H_2O$, then 0.1 M $NaHCO_3$ pH 8.0/0.5 M NaCl followed by 0.1 M NaAc pH 4.0/0.1 M NaCl and finally three times in buffer A (20 mM Hepes/NaOH pH 7.5, 150 mM NaCl, 0.25% Triton X-100, 1 mM EDTA, 1 mM EGTA). The Kinator I, II, III and IV beads were stored in the dark at 4°C as 1:1 suspension in buffer A plus 10 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM PMSF.

Synthesis of compound D (3-(5-Chloro-oxo-1,2-dihydro-indole-3-ylidenemethyl)-1*H*-indole-5-carboxylic acid(2-amino-ethyl)-amide



A 1 l flask equippped with a thermometer, reflux condenser and magnetic stirrer was charged with 1*H*-indole-5-carboxylic acid (25.0 g, 0.16 mol) and acetic acid (500 ml). Stirring was commenced and hexamethylenetetramine (43.5 g, 0.31 mol) was added. The mixture was heated to 95 to 100°C (internal temperature – the reaction solution changed colour from yellow to brown on heating). After 4 h (TLC reaction completion check; mobile phase; ethyl acetate; $R_{f(SM)}$ 0.55, $R_{f(Product)}$ 0.37; visualisation: UV-light 254 nm) the solution was allowed to cool to room temperature and quenched into water (2 l). The aqueous phase was extracted with ethyl acetate (3 x 700 ml), the combined extracts washed with saturated sodium chloride solution (700 ml), concentrated under vacuum (40°C, 250 – 80 mbar) and the crude product slurried in saturated sodium hydrogen carbonate solution (300 ml). The observed precipitate was collected by filtration, washed with acetone (50 ml) and dried under vacuum (45°C, 60 mbar, 3h). 3-Formyl-1*H*-indole-5-carboxylic acid was received as an off-white powder (16.7 g).

A 500 ml flask equipped with a magnetic stirrer and a thermometer was charged with methanol (315 ml) and 3-formyl-1*H*-indole-5-carboxylic acid (15.7 g, 82.9 mmol). Stirring was commenced and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride (25.3 g, 91.3 mmol) and (2-amino-ethyl)-carbamic acid *tert*-butyl ester (14.6 g, 91.3 mmol) were added in one portion. The mixture was stirred at room temperature for 5 h (TLC reaction completion check; mobile phase: ethyl acetate; $R_{f(SM)}$ 0.05, $R_{f(Product)}$ 0.25 -0.37; visualisation: UV-light 254 nm). The reaction mixture was quenched into saturated potassium carbonate solution (500 ml) and stirring was continued for 15 min. The aqueous phase was extracted with ethyl acetate (2 x 300 ml). The combined extracts were washed with 0.5 N hydrochloric acid (2 x 250 ml), water (200 ml) and dried over magnesium sulfate (40 g) for 30 min. The drying agent was removed by filtration and the solvent evaporated in vacuo (40°C, 180-80 mbar). The title compound was isolated as an off-white to light brown foam (20.3 g).

A 1 L flask equipped with a thermometer, reflux condenser and magnetic stirrer was charged with ethanol (400 ml) and {2-[3-formyl-1H-indole-5-carbonxyl)-amino]-ethyl}-carbamic acid *tert*-butyl ester (20.3 g, 61.2 mmol). Piperidine (40.6 ml, 34.9 g, 0.48 mol) and 5-chloro-1,3-dihydro-indol-2-one (12.3 g, 73.4 mmol) were added in one portion. The resulting suspension was heated to reflux for 5 h until no further change was observed by TLC (mobile phase: ethyl acetate; $R_{f(Chlorooxindole)}$ 0.53, $R_{f(Product)}$ 0.37; visualisation: UV-light: 254 nm). The mixture was allowed to cool to room temperature and ethanol (100 ml) was removed in vacuo

(40°C, 80-50 mbar). The observed solid was collected by filtration and washed with TBME (2 x 150 ml). The crude product was triturated with hot ethanol (100 ml), filtered, washed with ethanol (50 ml) and dried under vacuum (40 to 45°C, 3h) to afford the title compound as an orange solid (9.32 g, 19.3 mmol).

A 500 ml flask equipped with a magnetic stirrer and a gas outlet was charged with (2-{[3-(5-chloro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-1H-indole-5-carbonyl]-amino}-ethyl)-carbamic acid *tert* butyl ester (4.50 g, 9.36 mmol) and ethyl acetate (225 ml). Concentrated hydrochloric acid (9.00 ml) was added in one portion. The mixture was stirred for 60 h at room temperature ($^1$H-NMR reaction completion check). The solvent was removed in vacuo (40 to 45°C, 150-30 mbar). A second batch of the same size was prepared accordingly. The crude products from both batches were combined and slurried in methanol (200 ml). 25% Aqueous ammonia (5 ml) was added and the mixture stirred at room temperature for 15 min. The solids were collected by filtration, washed with methanol (2 x 50 ml) and dried under vacuum. The product was purified by column chromatography (silica gel: 300 g, mobile phase; dichloromethane / methane [95:5], gradient via dichloromethane / methanol / 25% aq. Ammonia [90:10:1] to [50:50:1]. The compound was obtained from the product fractions was slurried in methanol (200 ml) and filtered. The solvent was removed under vacuum (40 to 45°C, 100-40 mbar) to afford compound D, i.e. 3-(5-chloro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-1H-indole-5-carboxylic acid (2-amino-ethyl)-amide (3.54 g).

Preparation of cell lysates

10 cm petri dishes of confluent HuH-7 or HeLa cells were washed with 10 ml cold PBS and lysed on ice for 3 min in 300 µl lysis buffer per dish containing 20 mM Hepes/NaOH pH 7.5, 150 mM NaCl, 0.25% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF, 1 mM $Na_3VO_4$, 10 mM NaF, 1 mM DTT. Lysates were finally cleared by centrifugation (30 min, 13000 rpm, 4°C) and subsequently used for experiments.

Purification of kinases by in batch affinity chromatography using Kinator beads

200 µl of freshly prepared HuH-7 cell lysate was added to 20 µl drained Kinator I, II, III or IV beads. After incubation for 3 h at 4°C in the dark with continual agitation the supernatant was collected and the beads were washed three times in 200 µl buffer A (20 mM Hepes/NaOH pH 7.5, 150 mM NaCl, 0.25% Triton X-100, 1 mM EDTA, 1 mM EGTA). Finally the bound

kinases were eluted by incubation in 50 µl 1.5x SDS sample buffer for 3 min at 95°C. Alternatively, the beads were eluted in 200 µl buffer A containing 1 mM compound A, B, C or D, 10 mM ATP and 20 mM $MgCl_2$ for 2 h at 4°C in the dark with continual agitation. 10 µl supernatant and 10 µl eluent, respectively, were separated by SDS-polyacrylamidegelelectrophoresis (SDS PAGE) and transferred onto nitrocellulose membrane filters by semidry blotting or wet tank blotting. The kinases were detected by kinase specific antibodies and visualized using HRP conjugated secondary antibodies and the ECL detection system.

Purification of kinases by column affinity chromatography using Kinator I beads

1 ml of freshly prepared HuH-7 cell lysate was loaded onto a chromatography column filled with 250 µl Kinator I beads equilibrated to buffer A (20 mM Hepes/NaOH pH 7.5, 150 mM NaCl, 0.25% Triton X-100, 1 mM EDTA, 1 mM EGTA). The column dimensions were 12.5 mm x 5 mm and the flow rate was 50 µl/min. After washing the column with 8 volumes of buffer A, the kinases were eluted with 1 ml buffer A containing 1 mM compound A, 10 mM ATP and 20 mM $MgCl_2$. Flow through, washing fractions and eluents were collected in 1 ml fractions. 10 µl aliquots of each fraction were separated by SDS-PAGE and transferred onto nitrocellulose membrane filters by semidry blotting. Protein kinase p38 (p38 MAP kinase) was detected by specific antibodies and visualized using HRP conjugated secondary antibodies and the ECL detection system.

Purification of kinases by column affinity chromatography using Kinator I beads in the presence of high salt conditions

$2.5 \times 10^9$ HeLa-cells were lysed in 30 ml lysis buffer for 30 min on ice. After centrifugation the supernatant was adjusted to 1 M NaCl filtrated through a 0.45 µm cellulose acetate filter and loaded onto a chromatography column filled with 600 µl Kinator I beads equilibrated to high salt buffer (20 mM Hepes/NaOH pH 7.5, 1 M NaCl, 0.25% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM DTT). The column dimensions were 12.5 mm x 5 mm and the flow rate was 100 µl/min. After washing the column with 15 volumes of high salt buffer , the column was equilibrated with 2 ml low salt buffer (20 mM Hepes/NaOH pH 7.5, 150 mM NaCl, 0.25% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) and the kinases were eluted with 2 ml low salt buffer containing 1 mM compound A, 10 mM ATP and 20 mM $MgCl_2$. Equal aliquots (1/5000 of total amount) of cell lysate, flow through, wash and elution

fractions were separated by SDS-PAGE, transferred by semidry blotting onto nitrocellulose membrane filters and stained with Ponceau S. Protein kinases p38 (p38 MAP kinase) and RICK (RIP-like interacting CLARP kinase) were detected by specific antibodies and visualized using HRP conjugated secondary antibodies and the ECL detection system. In addition 1/500000 volume of cell lysate, flow through and increasing amounts of eluate were separated by SDS-PAGE and silver stained to demonstrate the enrichment factor.

Results

Compounds A, B, C, and D were covalently coupled to epoxy-activated Sepharose as described above resulting in the generation of the novel Kinator I, II, III, and IV chromatography media. For generation of the control matrix, ethanolamine instead of the compounds A, B, C or D was added to the coupling reaction. Total cell lysates from HuH-7 cells were prepared and added to either control beads or Kinator beads. After rotating the samples at 4°C, aliquots of the supernatant fractions were taken and the beads were then washed to remove unbound protein prior to elution of bound proteins with SDS sample buffer. After SDS-PAGE and transfer onto nitrocellulose, filters were probed with several antibodies that specifically recognize distinct protein kinases.

As shown in Fig. 1A, Kinator I beads containing the immobilised pyridinylimidazole derivative compound A particularly bound the protein kinases RICK (RIP-like interacting CLARP kinase) and p38 (p38 MAP kinase), as analysed by immunoblotting with specific antibodies. In stark contrast, none of these kinases showed any detectable binding to the control beads. Both RICK and p38 were depleted from the supernatant fractions in a way that correlated with their binding to Kinator I beads. Thus, these results clearly demonstrate that a functional immobilization of compound A could be achieved in a way that retains the ability of the compound to bind certain protein kinases from total cellular extracts.

As shown in Fig. 1B, Kinator II beads containing the immobilised pyridopyrimidine derivative compound B strongly bound the protein kinases Wee1 (Wee 1 tyrosine kinase) and MEK1 (MAP kinase kinase-1), as analysed by immunoblotting with specific antibodies. In stark contrast, none of these kinases showed any detectable binding to the control beads. Both Wee1 and MEK1 were depleted from the supernatant fractions in a way that correlated with their binding to Kinator II beads. Thus, these results clearly demonstrate that functional

immobilization of compound B could be achieved in a way that retains the ability of this compound to bind certain protein kinases from total cellular extracts.

As shown in Fig. 1C, Kinator III beads containing the immobilised bisindolylmaleimide derivative compound C strongly bound the protein kinases GSK3 (glycogen synthase kinase-3) alpha and beta, as analysed by immunoblotting with specific antibodies. In stark contrast, neither of the GSK3 isoforms alpha and beta showed any detectable binding to the control beads. Both isoforms were depleted from the supernatant fractions in a way that correlated with their binding to Kinator III beads.

Fig. 1D shows a similar type of experiment in which PKC (protein kinase C) alpha binding to Kinator III beads was tested in the presence or absence of PKC-specific cofactors (100 µg/ml phosphatidylserine, 20 µg/ml diacylglycerol and 150 µM $CaCl_2$). Interestingly, only when PKC alpha had been transferred into an active state due to co-factor addition, a strong and specific interaction with the Kinator III beads correlating with PKC alpha depletion from the respective supernatant fraction was observed, indicating that the Kinator III matrix can discern between active and inactive protein kinases as found for PKC alpha. Further experiments revealed that Kinator I beads can also bind one GSK3 isoform and Kinator II beads were found to deplete p38 from cellular extracts.

Thus, the four novel affinity reagents Kinator I, II, III and IV bind overlapping sets of protein kinases. Taken together, it is concluded that the combined use of the Kinator I, II, III, and IV media will significantly facilitate the identification of protein kinase targets since they show a strong affinity to at least one of the novel affinity reagents.

In the experiments shown above, protein kinases bound to Kinator I, II, III or IV were eluted with SDS sample buffer under denaturing conditions. To test whether milder elution conditions might be sufficient to quantitatively release bound kinases from Kinator beads, Kinator I beads were incubated after *in vitro* association with HuH-7 cell lysate with elution buffer containing either free compound A, ATP or compound A plus ATP. As shown in Fig. 2, neither addition of compound A nor ATP alone to the elution buffer lead to an efficient release of the bound protein kinase p38 from Kinator I beads. But, strikingly, when both compound A and ATP were added to the elution buffer, quantitative elution of p38 was observed. By this finding it was possible to adapt the novel Kinator beads to column

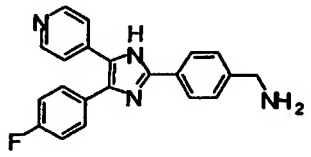
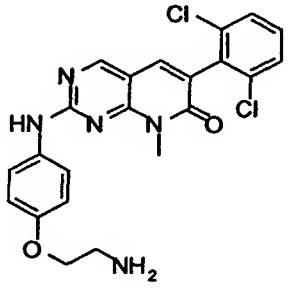
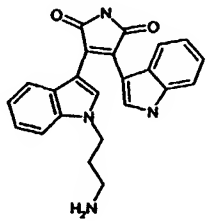
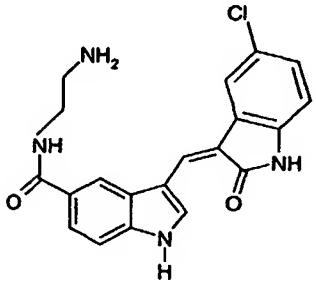
chromatographical applications, which require non-destructive elution conditions as a prerequisite for reusability of affinity columns. As demonstrated in Fig. 3, p38 from HuH-7 total cell lysate was efficiently retained on a Kinator I affinity column, remained bound during subsequent washing steps to remove unbound components, and could then be quantitatively eluted with buffer containing both compound A and ATP. As shown in the lower panel of Fig. 3, p38 was at least 100-fold enriched in the elutate when compared to the crude cell lysate as judged by Ponceau S staining. Application of this purification strategy to other affinity media featuring immobilized protein kinase inhibitors will be of general utility for chromatographical pre-enrichment of protein kinases, which is a prerequisite for subsequent target identification by proteome analysis focusing on this important class of potential drug targets.

A major improvement in the application of Kinator affinity media could be achieved by using high salt conditions (e.g. 1 M NaCl). As indicated in Fig. 4, Kinator I affinity column chromatography was performed in the presence of high salt conditions and equal aliquots of cell lysate. Lysate, flow through, washing and elution fractions were separated by SDS-PAGE. Western blotting with subsequent Ponceau S staining revealed that the flow through fraction looked just like the cell lysate fraction, whereas no visible amounts of protein could be detected in the washing and elution fractions. This demonstrates that non-specific interactions of protein components with the affinity matrix were highly reduced in the presence of high salt concentrations. Immunoblots using antibodies specific for the protein kinases p38 (p38 MAP kinase) and RICK (RIP-like interacting CLARP kinase), respectively, indicated that these kinases were fully depleted from cell lysate, remained bound during washing steps and eluted quantitatively in the presence of ATP and compound A.

For demonstration of the enrichment factor, increasing multiples of Kinator I eluate compared to cell lysate, flow through and washing fractions were separated by SDS-PAGE and silver stained. As shown in Fig. 5, proteins were enriched at least 10000-fold ($10^4$-fold). According to enhanced specificity a larger amount of cell lysate used as the starting material could be loaded onto the Kinator affinity chromatography column, resulting in the ability to detect also kinases of lower abundance. Proteins binding with high affinity to the Kinator matrix could be enriched by this method up to $10^6$-fold.

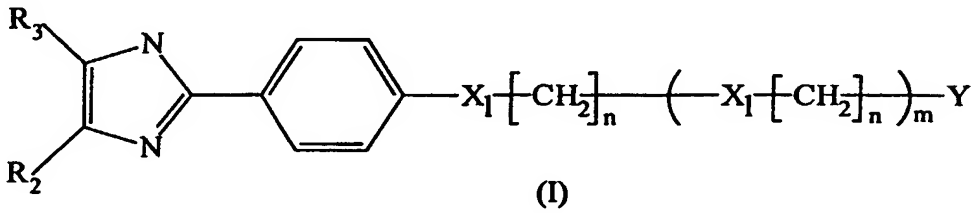
In Table 1 below, the compounds used to isolate protein kinases from a pool of proteins are given with their names and structural formulas.

Table 1: Compounds A to D

| Compound | name | structure |
|---|---|---|
| A | 4-[4-(4-Fluoro-phenyl)-5-pyridin-4-yl-1*H*-imidazol-2-yl]-benzylamine |  |
| B | 2-[4-(-Amino-ethoxy)-phenylamino]-6-(2,6-dichloro-phenyl)-8-methyl-8*H*-pyrido[2,3-d]pyrimidin-7-one |  |
| C | 2-[1-(3-Aminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide |  |
| D | 3-(5-Chloro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-1*H*-indole-5-carboxylic acid (2-amino-ethyl)-amide |  |

## Claims

1. A medium for separating at least one ATP binding protein from a pool of proteins, the medium comprising at least one compound of the general formula I



(I)

wherein

X is $-CH_2-$,-NH-, -O-, or –S-,

Y is $-NH_2$, $-NHR_1$, -OH or –SH,

l is 0 or 1,

m is an integer from 0 to 10,

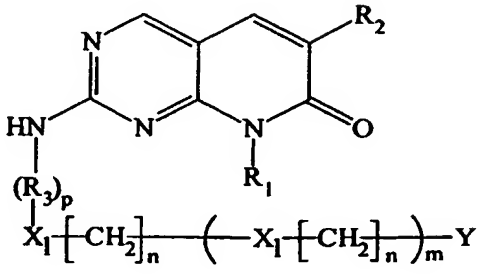
n is an integer from 0 to10,

$R_1$ is $C_1 - C_6$ alkyl (linear or branched) or aryl,

$R_2$ is unsubstituted or partially or fully substituted aryl, substituted by -F, -Cl, -Br, -I, -CN, -OH, -SH- , $C_1 - C_6$-alkoxy, $C_1 - C_6$ -alkylthio, $C_1 - C_6$ -haloalkyloxy, $C_1$-$C_6$ haloalkyl ($C_1 - C_6$-alkoxy denotes an O-alkyl group, $C_1 - C_6$-alkylthio denotes an S-alkyl group, $C_1 - C_6$-haloalkyloxy denotes an halogen-alkyl-O group, $C_1 - C_6$-haloalkyl denotes an halogen-alkyl group wherein the alkyl group is linear or branched), and

$R_3$ is pyridinyl or pyrimidinyl;

formula II



(II)

wherein

$R_1$ is $C_1 - C_6$ alkyl (linear or branched) or aryl,

$R_2$ and $R_3$ are unsubstituted or partially or fully substituted aryl substituted by -F, -Cl, -Br, -I, -CN, -OH, -SH- , $C_1 - C_6$-alkoxy, $C_1 - C_6$ -alkylthio, $C_1 - C_6$ -haloalkyloxy, $C_1$-$C_6$ haloalkyl ($C_1 - C_6$-alkoxy denotes an O-alkyl group, $C_1 - C_6$-alkylthio denotes an S-alkyl group, $C_1 - C_6$-haloalkyloxy denotes an halogen-alkyl-O group, $C_1 - C_6$-haloalkyl denotes an halogen-alkyl group wherein the alkyl group is linear or branched),

X is -$CH_2$-,-NH-, -O-, -S-,

Y is -$NH_2$, -$NHR_1$, -OH or –SH,

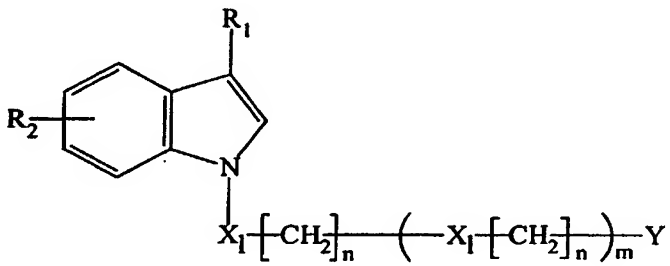
l is 0 or 1,

m is 0 an integer from 0 to 10, and

n is an integer from 0 to10 and

p is an integer from 0 to 2;

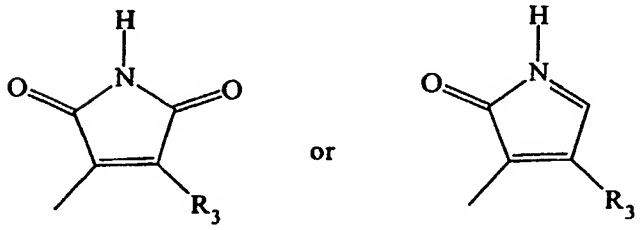
formula III



(III)

wherein

$R_1$ is



$R_3$ is -indolyl, N-alkyl indolyl, -$NHR_1$', -S-$R_1$', -O-$R_1$',

$R_1$' is $C_1 - C_6$ alkyl (linear or branched) or aryl,

$R_2$ is -H, -F, -Cl, -Br, -I, -CN, -OH, -SH- , $C_1 - C_6$-alkoxy, $C_1 - C_6$ -alkylthio, $C_1 - C_6$ -haloalkyloxy, $C_1$ - $C_6$ haloalkyl ($C_1 - C_6$-alkoxy denotes an O-alkyl group, $C_1 - C_6$-alkylthio denotes an S-alkyl group, $C_1 - C_6$-haloalkyloxy denotes an halogen-alkyl-O group, $C_1 - C_6$-haloalkyl denotes an halogen-alkyl group wherein the alkyl group is linear or branched),

X is $-CH_2-$,-NH-, -O-, or –S-,

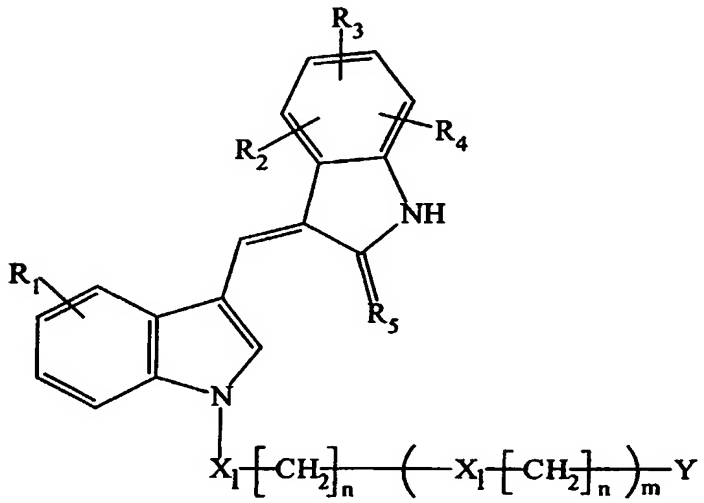
Y is $-NH_2$, $-NHR_1$, -OH or –SH,

l is 0 or 1,

m is 0 an integer from 0 to 10, and

n is an integer from 0to 10;

or formula IV



(IV)

wherein

X is $-CH_2-$,-NH-, -O-, or –S-,

Y is $-NH_2$, -NHR', -OH or –SH,

Z is $-CH_2$, -CO, -O-CO, -N-CO, $-OCH_2$, or $-SCH_2$,- CO-O, -CO-N

l is 0 or 1,

m is an integer from 0 to10,

n is an integer from 0 to10,

$R_1$ is $-Z-NH-(CH_2)_n-[X_l-(CH_2)_n]_m-Y$,

R' is $C_1 - C_6$ alkyl (linear or branched) or aryl,

$R_2$, $R_3$, and $R_4$ represent independently of each other -H, -F, -Cl, -Br, -I, -CN, -OH, -SH- , $C_1 - C_6$-alkoxy, $C_1 - C_6$ -alkylthio, $C_1 - C_6$ -haloalkyloxy, $C_1$-$C_6$ haloalkyl ($C_1 - C_6$-alkoxy denotes an O-alkyl group, $C_1 - C_6$-alkylthio denotes an S-alkyl group, $C_1 - C_6$-haloalkyloxy denotes an halogen-alkyl-O group, $C_1 - C_6$-haloalkyl denotes an halogen-alkyl group wherein the alkyl group is linear or branched), and
$R_5$ is S or O,

immobilized on a support material.

2. The medium according to claim 1, wherein at least one of the compounds 4-[4-(4-fluoro-phenyl)-5-pyridine-4-yl-1*H*-imidazole-2-yl]-benzylamine, 2-[4-(amino-ethoxy)-phenylamino]-6-(2,6-dichloro-phenyl)-8-methyl-8*H*-pyrido[2,3-d]pyrimidine-7-one, 2-[1-(3-aminopropyl)-1*H*-indole-3-yl)maleimide and 3-(5-chloro-2-oxo-1,2-dihydro-indole-3-ylidenemethyl)-1*H*-indole-5-carboxylic acid (2-amino-ethyl)-amide is immobilized on the support material.

3. The medium according to claim 1 or 2, wherein the compounds are covalently bound to the support material.

4. The medium according to one of the preceding claims, wherein the support material comprises agarose.

5. The medium according to claim 4, wherein the agarose is a modified agarose material.

6. The medium according to one of the preceding claims, wherein the support material comprises ferro- or ferrimagnetic particles.

7. The medium according to claim 6, wherein the ferro- or ferrimagnetic particles are made from glass or plastic.

8. The medium according to claim 6 or 7, wherein the ferro- or ferrimagnetic particles are porous.

9. The medium according to claim 8, wherein the ferro- or ferrimagnetic particles have a surface area of about 190 g/m² or greater, determined according the BET method.

10. The medium according to one of claims 6 to 9, wherein the ferro- or ferrimagnetic particles comprise about 30 to 50 % by weight of $Fe_3O_4$ and about 50 to 70 % by weight of $SiO_2$.

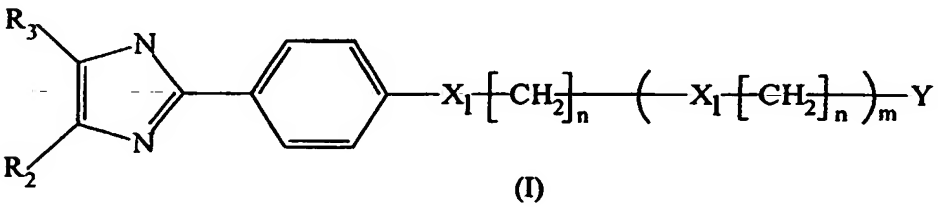
11. The medium according to one of claims 6 to 10, wherein the average size of the ferro- or ferrimagnetic particles is from 5 to 25 µm in diameter.

12. The medium according to one of the preceding claims, wherein the pool of proteins is a proteome, a cell lysate or a tissue lysate.

13. The medium according to one of the preceding claims, wherein the ATP binding protein is a protein kinase.

14. A method for enriching, purifying or depleting at least one ATP binding protein from a pool of proteins containing at least one ATP binding protein, the method comprising the following steps:

a) immobilizing at least one compound of the general formula I



(I)

wherein

X is $-CH_2-$, -NH-, -O-, or –S-,

Y is $-NH_2$, $-NHR_1$, -OH or –SH,

l is 0 or 1,

m is an integer from 0 to 10,

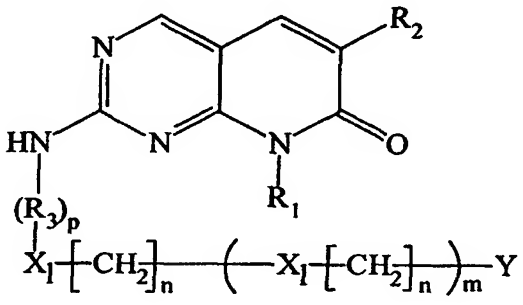
n is an integer from 0 to 10,

$R_1$ is $C_1 - C_6$ alkyl (linear or branched) or aryl,

$R_2$ is unsubstituted or partially or fully substituted aryl, substituted by -F, -Cl, -Br, -I, -CN, -OH, -SH- , $C_1$ – $C_6$-alkoxy, $C_1$ – $C_6$ -alkylthio, $C_1$ – $C_6$ -haloalkyloxy, $C_1$-$C_6$ haloalkyl ($C_1$ – $C_6$-alkoxy denotes an O-alkyl group, $C_1$ – $C_6$-alkylthio denotes an S-alkyl group, $C_1$ – $C_6$-haloalkyloxy denotes an halogen-alkyl-O group, $C_1$ – $C_6$-haloalkyl denotes an halogen-alkyl group wherein the alkyl group is linear or branched), and

$R_3$ is pyridinyl or pyrimidinyl;

formula II



(II)

wherein

$R_1$ is $C_1$ – $C_6$ alkyl (linear or branched) or aryl,

$R_2$ and $R_3$ are unsubstituted or partially or fully substituted aryl Substituted by -F, -Cl, -Br, -I, -CN, -OH, -SH- , $C_1$ – $C_6$-alkoxy, $C_1$ – $C_6$ -alkylthio, $C_1$ – $C_6$ -haloalkyloxy, $C_1$-$C_6$ haloalkyl

($C_1$ – $C_6$-alkoxy denotes an O-alkyl group, $C_1$ – $C_6$-alkylthio denotes an S-alkyl group, $C_1$ – $C_6$-haloalkyloxy denotes an halogen-alkyl-O group, $C_1$ – $C_6$-haloalkyl denotes an halogen-alkyl group wherein the alkyl group is linear or branched),

X is -$CH_2$-,-NH-, -O-, -S-,

Y is -$NH_2$, -$NHR_1$, -OH or –SH,

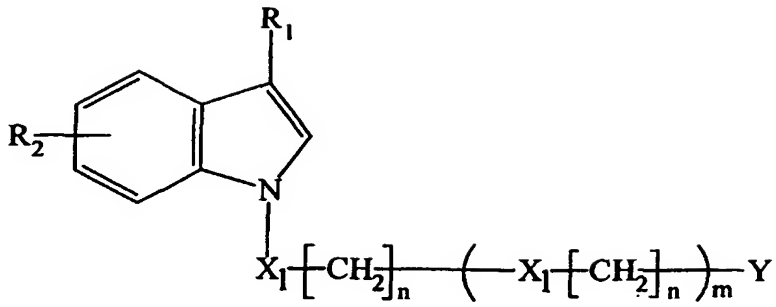
l is 0 or 1,

m is an integer from 0 to 10,

n is an integer from 0 to 10, and

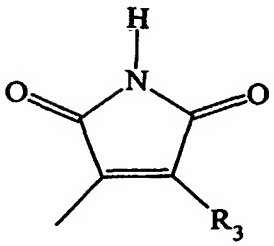
p is an integer from 0 to 2;

formula III

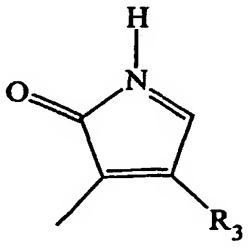


wherein (III)

$R_1$ is



or



$R_3$ is -indolyl, N-alkylindolyl, $-NHR_1'$, $-S-R_1'$, $-O-R_1'$,

$R_1'$ is $C_1 - C_6$ alkyl (linear or branched) or aryl,

$R_2$ is -H, -F, -Cl, -Br, -I, -CN, -OH, -SH- , $C_1 - C_6$-alkoxy, $C_1 - C_6$ -alkylthio, $C_1 - C_6$ -haloalkyloxy, $C_1$ - $C_6$ haloalkyl ($C_1 - C_6$-alkoxy denotes an O-alkyl group, $C_1 - C_6$-alkylthio denotes an S-alkyl group, $C_1 - C_6$-haloalkyloxy denotes an halogen-alkyl-O group, $C_1 - C_6$-haloalkyl denotes an halogen-alkyl group wherein the alkyl group is linear or branched),

X is $-CH_2-$,-NH-, -O-, or –S-,

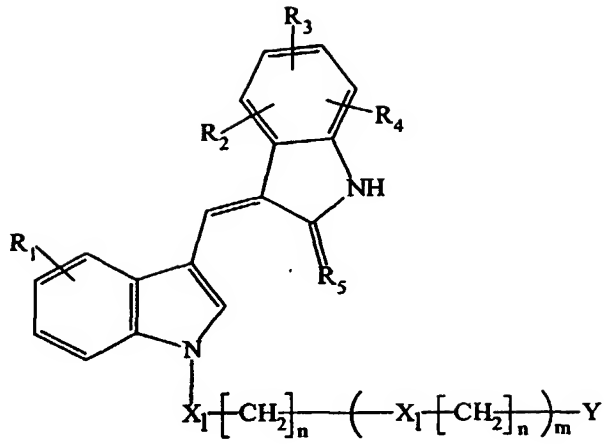
Y is $-NH_2$, $-NHR_1$, -OH or –SH,

l is 0 or 1,

m is an integer from 0 to 10, and

n is an integer from 0 to 10;

or formula IV



(IV)

wherein

X is $-CH_2$,-NH, -O, or –S,

Y is $-NH_2$, -NHR', -OH or –SH,

Z is $–CH_2$, CO, -O-CO, -N-CO, $-OCH_2$, or $-SCH_2$, -CO-O, -CO-N,

l is 0 or 1,

m is an integer from 0 to 10,

n is an integer from 0 to 10,

$R_1$ is $-Z-NH-(CH_2)_n-[X_l-(CH_2)_n]_m-Y$,

R' is $C_1 – C_6$ alkyl (linear or branched) or aryl,

$R_2$, $R_3$, and $R_4$ represent independently of each other -H, -F, -Cl, -Br, -I, -CN, -OH, -SH- , $C_1 – C_6$-alkoxy, $C_1 – C_6$ -alkylthio, $C_1 – C_6$ -haloalkyloxy, $C_1$-$C_6$ haloalkyl ($C_1 – C_6$-alkoxy denotes an O-alkyl group, $C_1 – C_6$-alkylthio denotes an S-alkyl group, $C_1 – C_6$-haloalkyloxy denotes an halogen-alkyl-O group, $C_1 – C_6$-haloalkyl denotes an halogen-alkyl group wherein the alkyl group is linear or branched), and

$R_5$ is S or O,

on a support material;

b) bringing the pool of proteins containing at least one protein kinase into contact with at least one of the compounds immobilized on the support material; and

c) separating the proteins not bound to the at least one compound immobilized on the support material from the at least one protein kinase bound to the at least one compound immobilized on the support material.

15. The method according claim 14, wherein the at least one compound immobilized on the support material is 4-[4-(4-fluoro-phenyl)-5-pyridine-4-yl-1*H*-imidazole-2-yl]-benzylamine, 2-[4-(amino-ethoxy)-phenylamino]-6-(2,6-dichloro-phenyl)-8-methyl-8*H*-pyrido[2,3-d]pyrimidine-7-one, 2-[1-(3-aminopropyl)-1*H*-indole-3-yl)maleimide or 3-(5-chloro-2-oxo-1,2-dihydro-indole-3-ylidenemethyl)-1*H*-indole-5-carboxylic acid (2-amino-ethyl)-amide.

16. The method according to claim 14 or 15, further comprising a step:

d) releasing the at least one protein kinase bound to the at least one compound immobilized on the support material from the at least one of said compounds.

17. The method according to claim 16, further comprising a step:

e) collecting the released at least one protein kinase.

18. The method according to one of claims 14 to 17, wherein the support material comprises agarose.

19. The method according to claim 18, wherein the agarose is a modified agarose material.

20. The method according to one of claims 14 to 17, wherein the support material comprises ferro- or ferrimagnetic particles.

21. The method according to one of claims 14 to 20, wherein in step c) the separating of the proteins not bound to the at least one compound immobilized on the support material from the at least one ATP binding protein bound to the at least one compound immobilized on the support material is effected by washing with a buffer containing 5 to 500 mM Hepes pH 6.5-8.5 or 5 to 500 mM Tris-HCl pH 6.8 to 9.0, 0 to 1000 mM NaCl, 0.0 to 5% Triton X-100, 0 to 500 mM EDTA, and 0 to 200 mM EGTA.

22. The method according to claim 21, wherein the buffer contains 20 mM Hepes/NaOH pH 7.5, 150 mM NaCl, 0.25% Triton X-100, 1 mM EDTA, and 1 mM EGTA.

23. The method according to one of claims 16 to 22, wherein in step d) the releasing of the at least one protein kinase bound to the at least one compound immobilized on the support material is effected by washing with a buffer containing 5 to 500 mM Hepes pH 6.5-8.5 or 5 to 500 mM Tris-HCl pH 6.8 to 9.0, 0 to 1000 mM NaCl, 0.0 to 5.0% Triton X-100, 0 to 500 mM EDTA, 0 to 200 mM EGTA, 1 to 100 mM ATP, 1 to 200 mM $MgCl_2$ and 0.1 to 10 mM of at least one of the compounds immobilized on the support material.

24. The method according to claim 23, wherein the buffer contains 20 mM Hepes pH 7.5, 150 mM NaCl, 0.25% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM ATP, 20mM $MgCl_2$ and 1 mM of at least one of the compounds immobilized on the support material.

25. The method according to one of claims 14 to 24, wherein the pool of proteins is a proteome, cell lysate or tissue lysate.

26. The method according to one of claims 14 to 25, wherein the ATP binding protein is a protein kinase.

27. The method according to one of claims 14 to 26, wherein the pool of proteins contains 0.5 to 5 M, preferably 0.5 to 3 M, and more preferably 0.75 to 2 M of a salt.

28. The method according claim 27, wherein the salt is an alkali metal salt.

29. The method according to claim 28, wherein the alkali metal salt is NaCl.

30. The method according to one of claims 14 to 29, wherein the at least one ATP binding protein is enriched at least 100-fold from the pool of proteins.

31. The method according to claim 30, wherein the at least one ATP binding protein is enriched between 100- and 1000-fold.

32. The method according to one of claims 28 or 29, wherein the at least one ATP binding protein is enriched at least $10^4$-fold and preferably up to $10^6$-fold.

33. Kit comprising a medium according to one of claims 1 to 13.

34. Kit according to claim 33, further comprising at least one buffer according to one of claims 21 to 24.

## Abstract

The present invention relates to a medium and a method for enriching ATP binding proteins, e.g. proteinkinases, from a pool of proteins, like a proteome. The medium of the present invention comprises specific inhibitors, e.g. at least one of the compounds 4-[4-(4-fluoro-phenyl)-5-pyridine-4-yl-1*H*-imidazole-2-yl]-benzylamine, 2-[4-(amino-ethoxy)-phenylamino]-6-(2,6-dichloro-phenyl)-8-methyl-8H-pyrido[2,3-d]pyrimidine-7-one, 2-[1-(3-aminopropyl)-1*H*-indole-3-yl)maleimide and 3-(5-chloro-2-oxo-1,2-dihydro-indole-3-ylidenemethyl)-1H-indole-5-carboxylic acid (2-amino-ethyl)-amide, immobilized on a support material. According to the method of the present invention the above-mentioned immobilized compounds are used to selectively bind protein kinases from a pool of heterogeneous proteins.

(Fig. 5)

Fig. 1 A

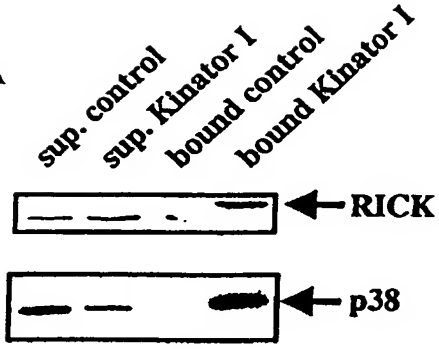



Fig. 1 B

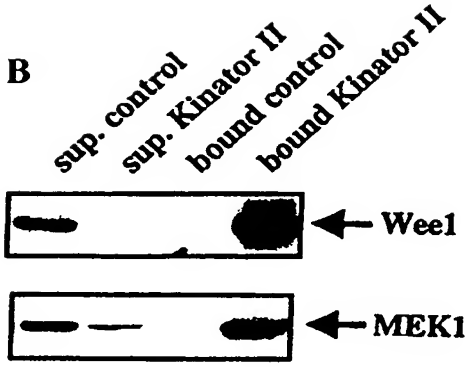



Fig. 1 C

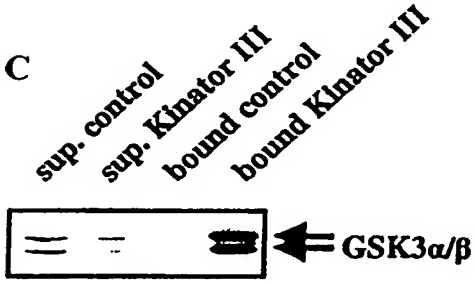
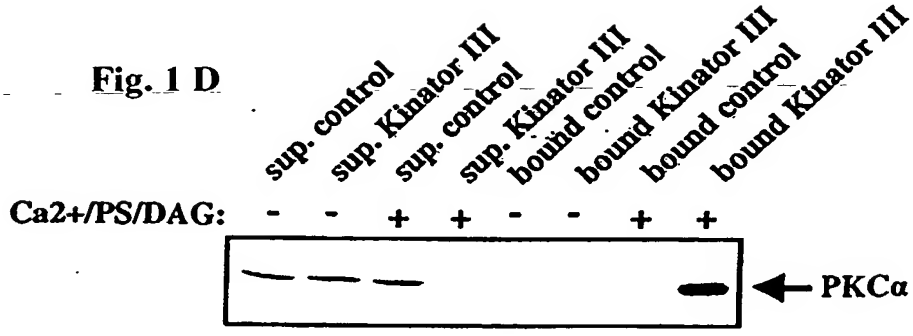



Fig. 1 D



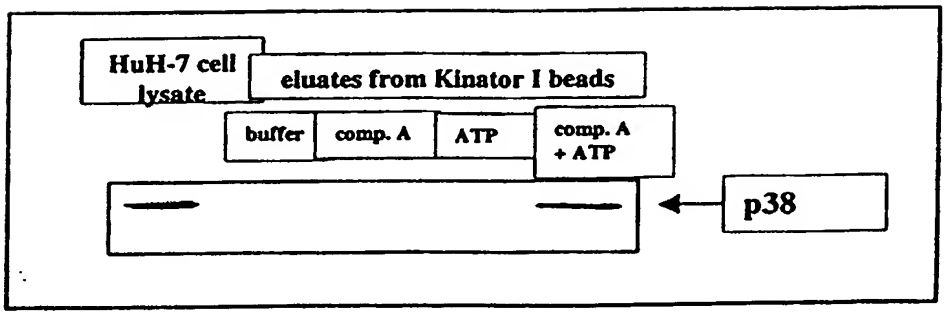



Fig. 2

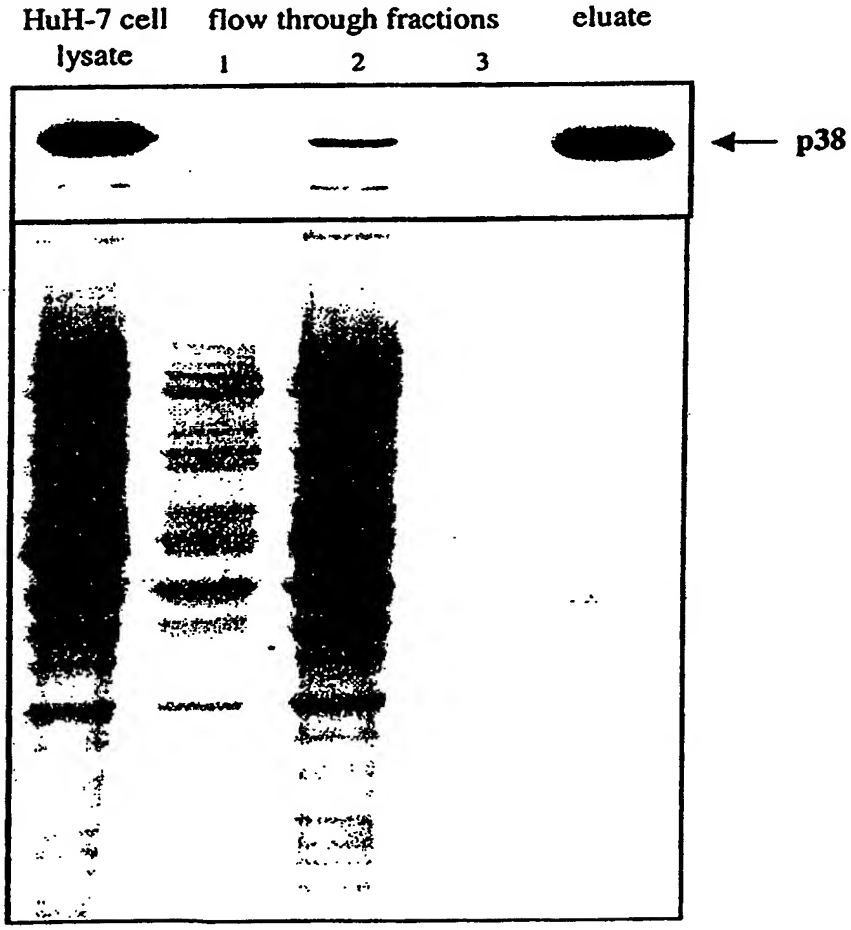



Fig. 3

HeLa-lysate on Kinator I column

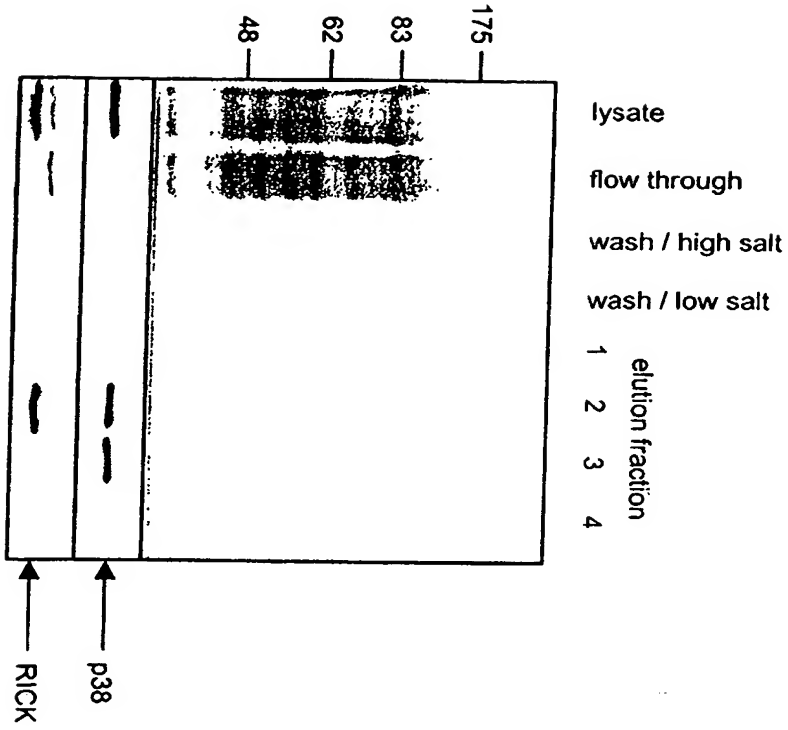



Fig. 4

Enrichment of proteins on Kinator I column

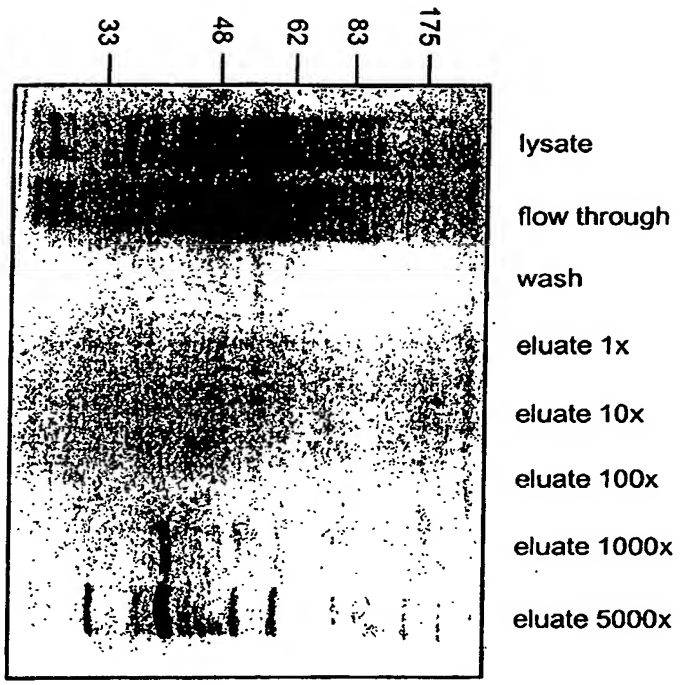



Fig. 5